Multidimensional HPLC; Concepts and Practical Applications

Part – 1:

Introduction, basic concepts of 2D-LC, what is peak capacity, definition of orthogonality, sampling in 2D LC, requirements to the 1st dimension separation, requirements to the 2nd dimension separation

About the Author

Microscale Separations and

- Undergraduate and graduate studies at University of Amsterdam, 1964-1976. Majors in Organic Chemistry and Chemical Engineering
- Post-doctoral research at State University of Ghent, Belgium, 1977 and post-doctoral training Analytical Chemistry, University of Amsterdam, 1978-1979
- R&D Chemist, group & project Leader, R&D section manager, HPLC column and HPLC system development at Hewlett-Packard, Waldbronn, Germany, 1979-'99
- Since 2000, Agilent Technologies University Relations and External Scientific Collaborations Manager, 2006 Agilent Technologies Research Fellow
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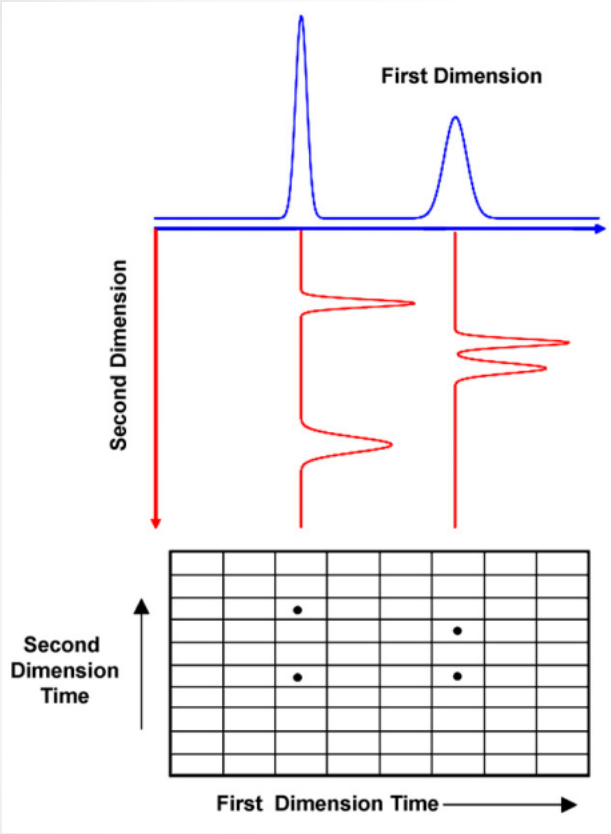
What is Multidimensional HPLC?



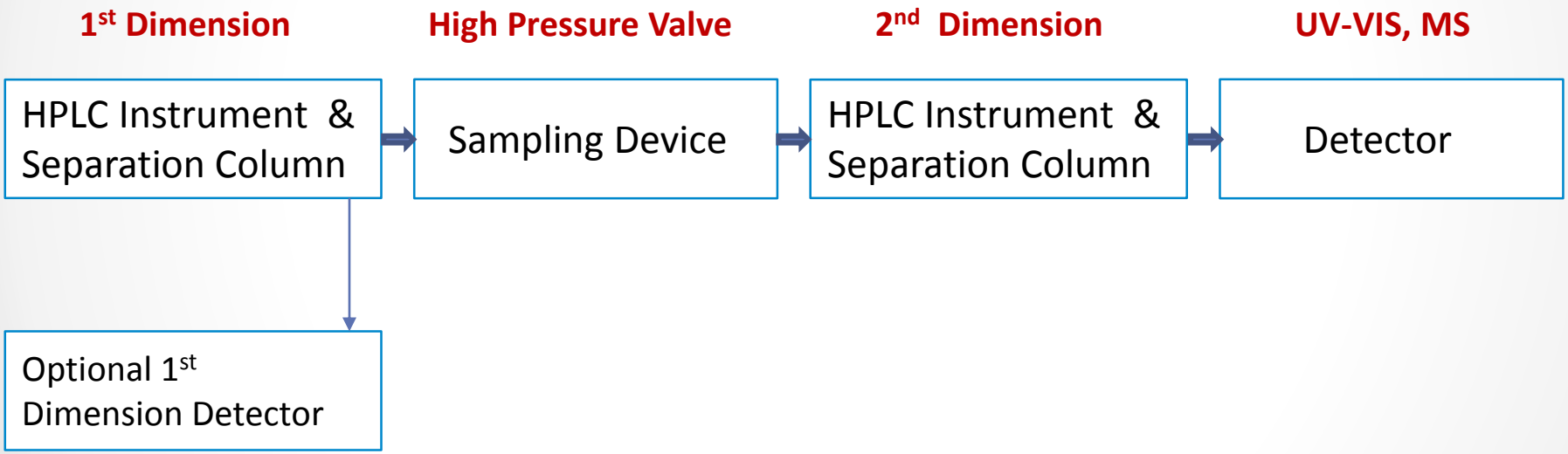
Peak capacity by the product of the number of bins

$$ {}^{1}Z_p * {}^{2}Z_p $$

What is Multidimensional HPLC?

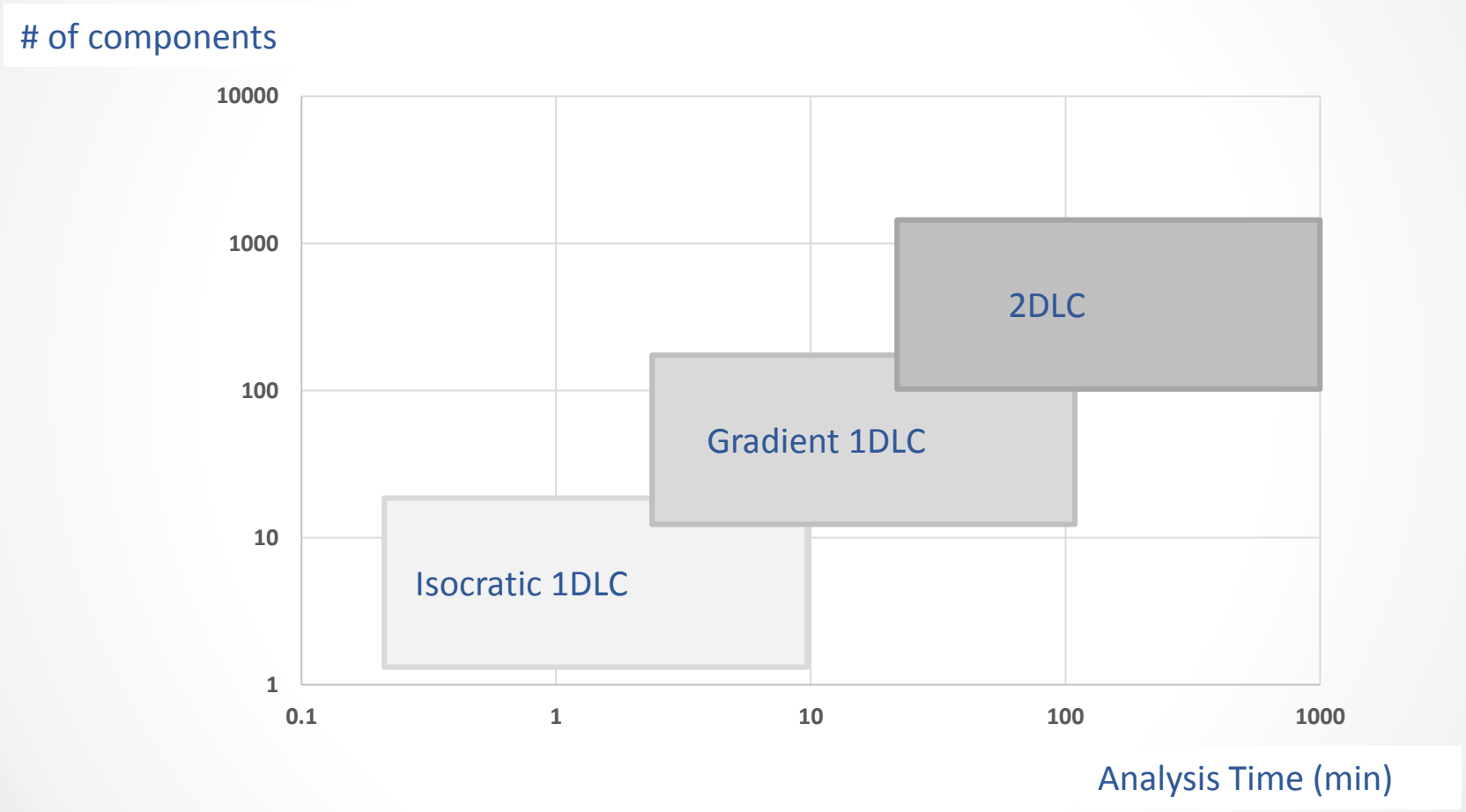
Simple Block Diagram

Microscale Separations and Bioanalysis



Positioning of HPLC Techniques[1,2]

Microscale Separations and Bioanalysis



Adapted from [1]Stoll, D., University of Minnesota Ph.D. Dissertation, 2007, [2]Stoll, D., *et al.*, *J. of Chrom. A*, 1168, 3 (2007)

Applications Areas of MDLC

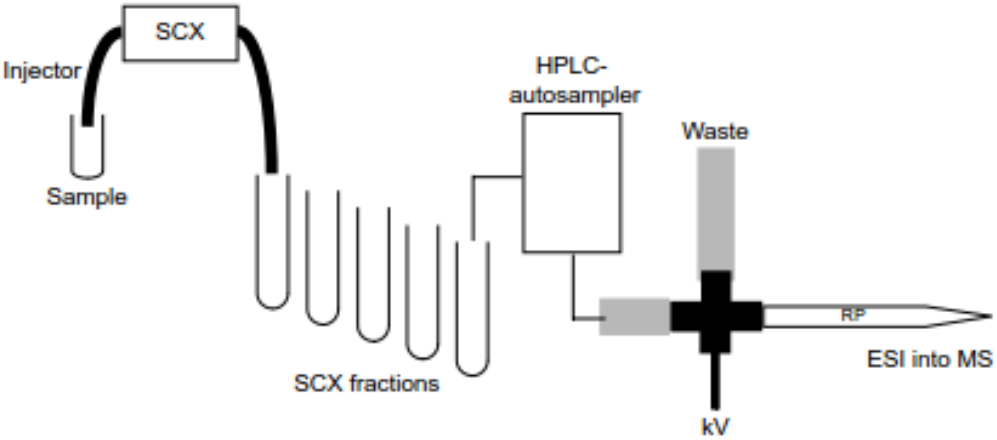
- Food, Beverages and Consumer Goods
  - Original Ingredients, Contamination, Proof of Authenticity
- Proteomics, Metabolomics
  - Life Science Research
  - Biomarker discovery
  - Biopharmaceutical (originator or biosimilars)
- Environmental Analysis
  - Identification of Pollutants, Contaminants, Cause of Accidents
- Chemical Industry
  - Polymers, Oligomers, Branching, Functional Group Analysis
- Forensics & Toxicology
  - Poison, Doping,
- Pharmaceutical Analysis
  - DMPK, metabolite identification
  - Traditional Chinese Medicine

Important Nomenclature

- LC-LC heart-cut two-dimensional liquid chromatography
- LCxLC comprehensive two-dimensional liquid chromatography
- ^{1}D 'first dimension'; for example, ^{1}D column means 'first dimension' column
- ^{2}D 'second dimension'; same ^{2}D column means 'second dimension' column
- 1D denotes a one-dimensional system
- 2D denotes a two-dimensional system e.g. 2D LC
- $^{1}t_r$ retention time for a given peak in the first dimension
- $^{1}t_0$ "dead" time of the first dimension conditions
- ^{1}k retention factor of a given compound eluting from the first dimension column
- ^{1}N the number of theoretical plates of the first dimension column
- ^{1}w width of a peak eluting from the first dimension column
- $^{1}R_S$ resolution of a peak pair eluting from the first dimension column
- $^{1}Z_p$ peak capacity of the first dimension column
- n_c number of components in the sample

Principle Methods of 2D LC

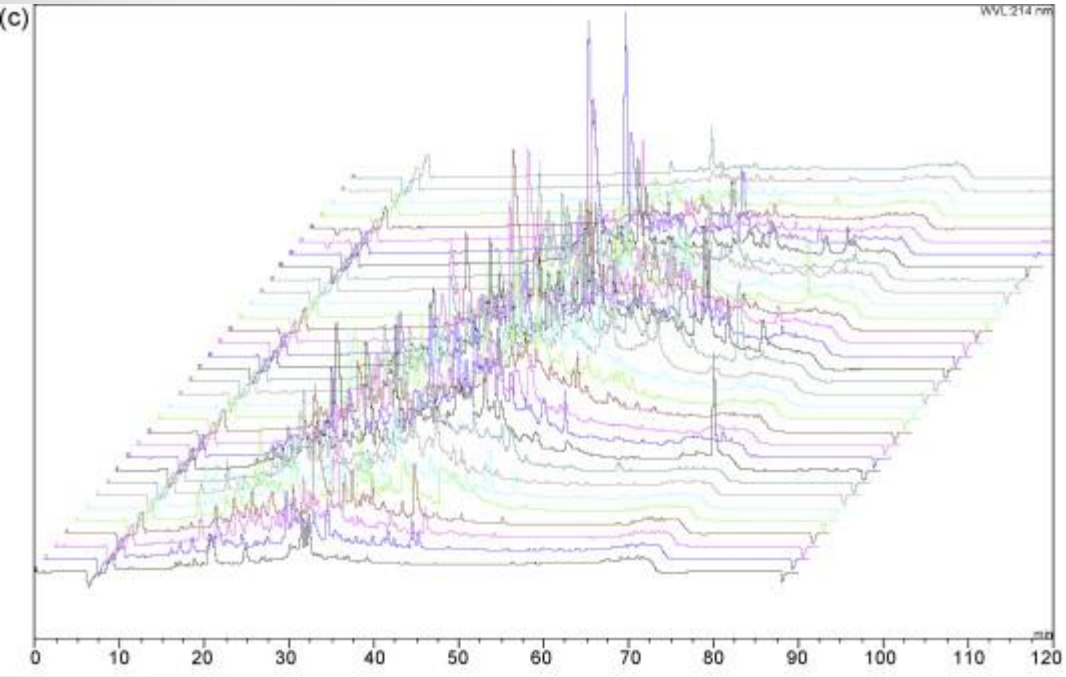
- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation column, store and re-inject in the 2nd dimension separation column in separate next run.



Picture taken from S.K. Swanson and M.P. Washburn, Drug Discovery Today, 10, 719 (2005)

Principle Methods of 2D LC

"Offline" approaches



1st dimension:
150 mm L x 2.1 mm ID x 3.5 µm XBridge phenyl column

Offline fraction collection and reinjection in the **2nd dimension**:
150 x 0.075 mm, 3 µm Pepmap 100Å C18 particles

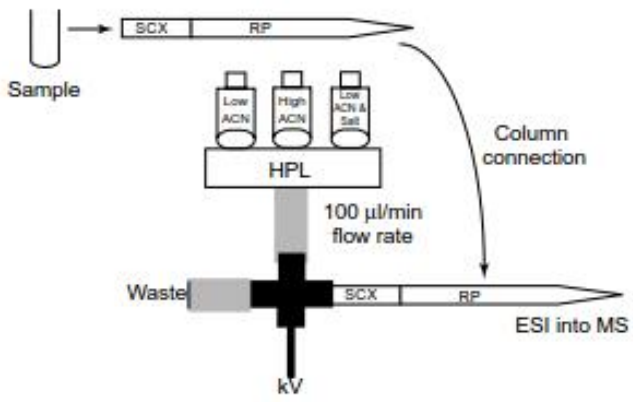
Total time required 40x1.5 hrs!!

K. Sandra et al., J. Chrom. B, 877, 1019 (2009)

Principle Methods of 2D LC

"Offline" approaches

- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation, store and re-inject in the 2nd dimension separation column later.
- "Stop-and-Go" methods e.g. MuDPIT*
  (Multi-Dimensional Protein Identification Technology)
  - One column packed with a segment of ion exchanger and a larger segment of RP-phase.
    A pulsed salt gradient in IEX displaces a fraction of the sample onto the RP-column



Picture taken from S.K. Swanson and M.P. Washburn, Drug Discovery Today, 10, 719 (2005)

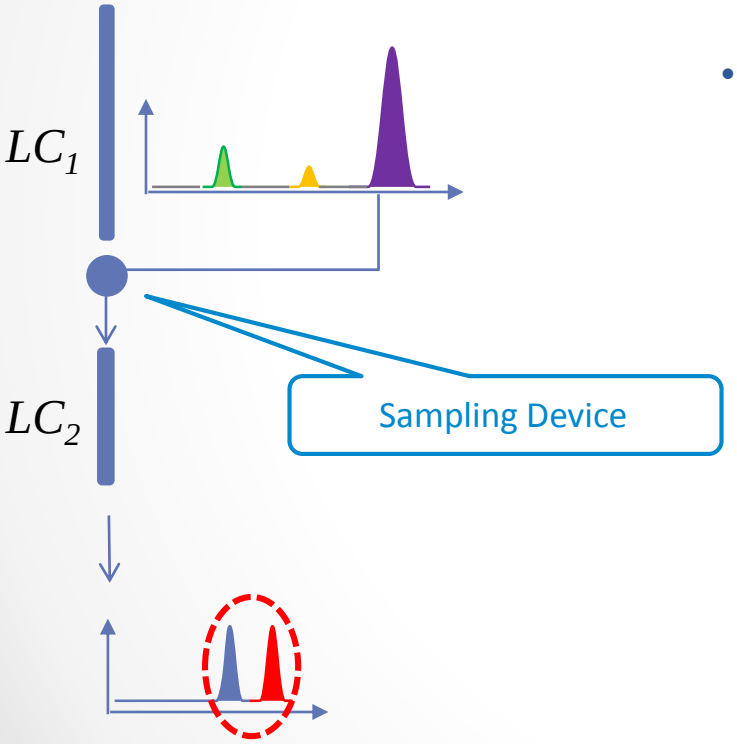
*J.R. Yates III et al., Int. J. of Mass Spectrometry 219 (2002) 245

Principle Methods of 2D LC

- "Offline" methods (sequential)
  - Collect fractions from the 1st dimension separation, store and re-inject in the 2nd dimension separation column later.
- "Stop-and-Go" methods (e.g. Multi-Dimensional Protein Identification Technology)
  - One column packed with a segment of ion exchanger and a larger segment of RP-phase. A pulsed salt gradient in IEX displaces a fraction of the sample onto the RP-column
- "On-line" methods (parallel)
  - **Heart-cut:**
    Selected fractions from the 1st dimension separation and intermediately stored on-line and delivered on-line to the 2nd dimension separation
  - **Comprehensive:**
    Fractions are continuously taken from the eluate from the 1st dimension separation, intermediately stored on-line and delivered to the 2nd dimension separation

Principle Methods of 2D LC

Heart-cutting LC-LC



- Selected fractions of the 1st dimension separation are injected onto the 2nd dimension column
  → 1st dimension detector optional
- Long 1st dimension gradient separation possible
  → good data quality, limited information

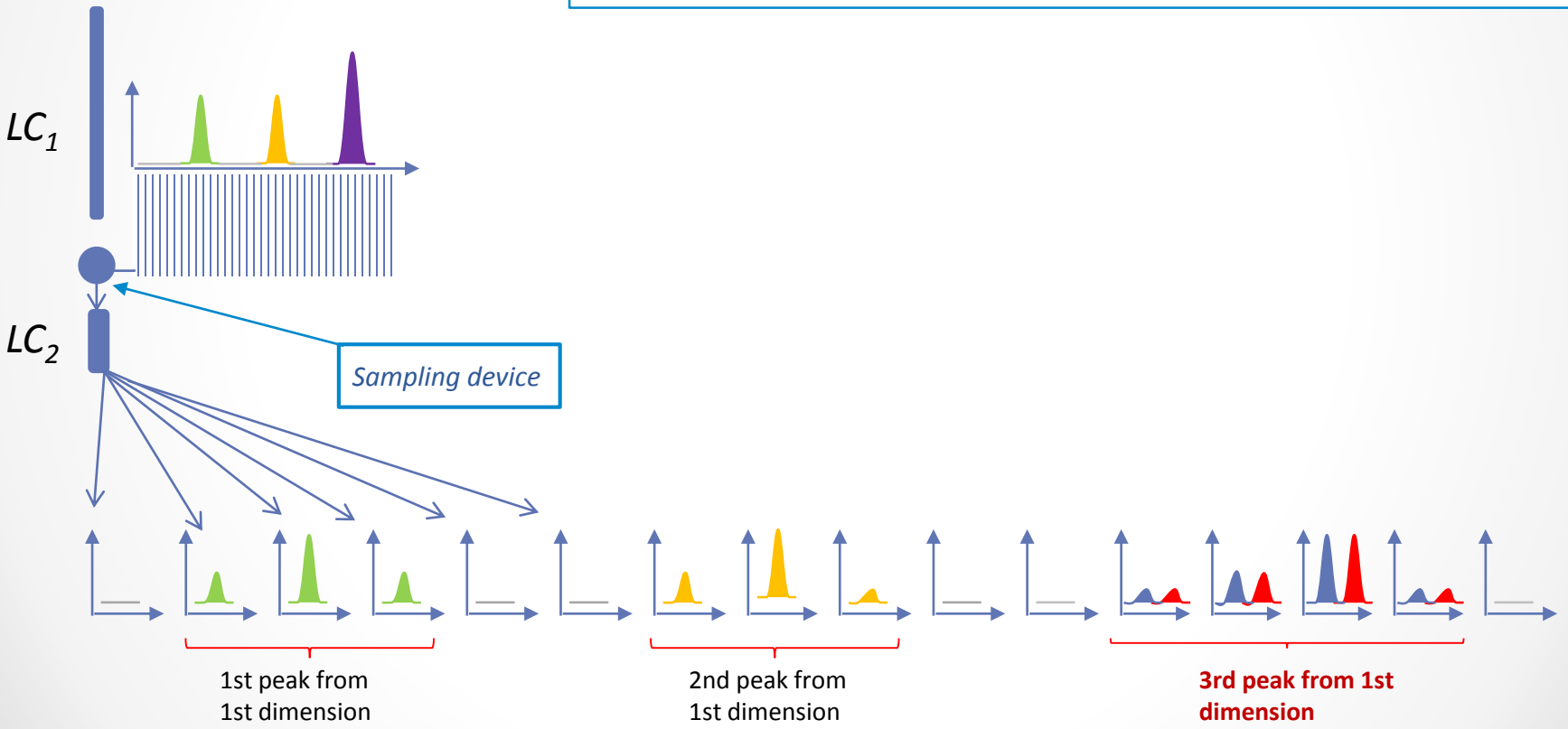
Slide courtesy of Agilent Technologies

Principle Methods of 2D LC

Comprehensive 2D-LC

Microscale Separations and Bioanalysis

- The **whole** ^{1}D effluent is continuously injected onto ^{2}D column
- In the 2nd dimension (ultra)short analysis (separation) time is necessary mandating high flow rate pumps & fast detection
- → Good data quality; full („comprehensive“) 2D information!



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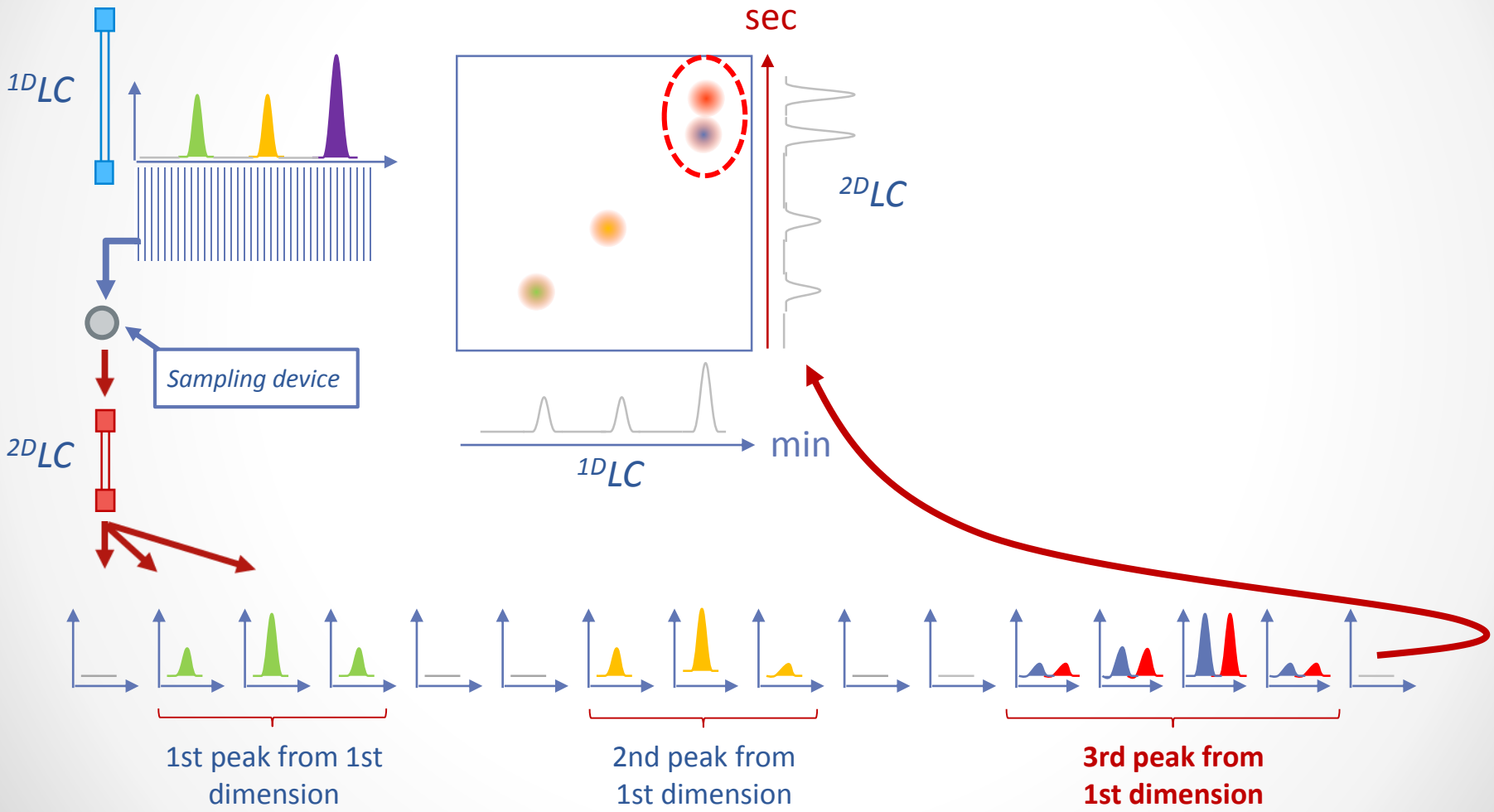
Principle Methods of 2D LC

Comprehensive 2D LC

Microscale Separations and Bioanalysis



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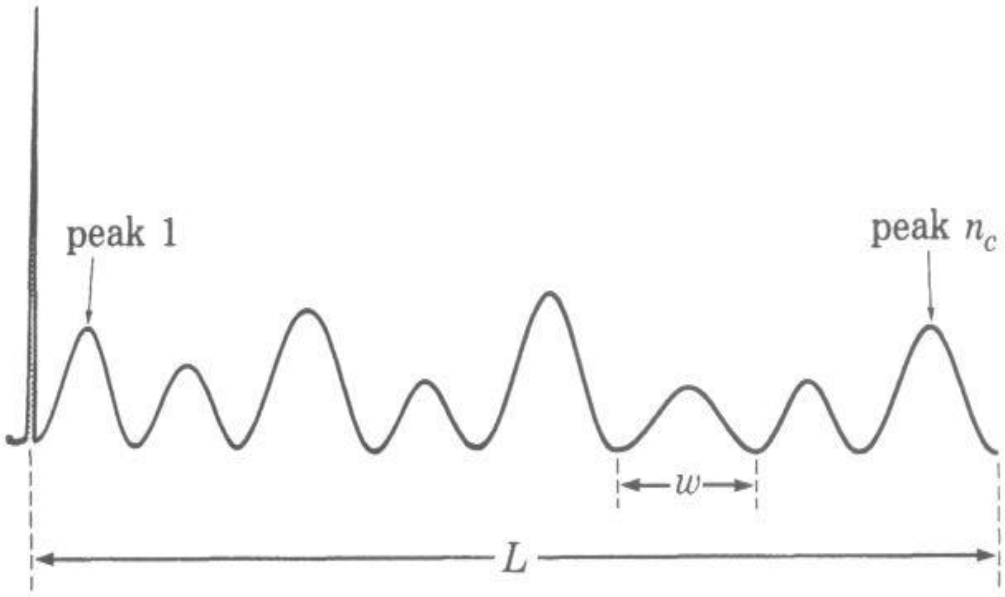
Peak Capacity in 1D and 2D HPLC

Peak Capacity (Z_p) – Ideal Case*



Peak capacity is defined as the number of peaks or zones Z_p that can be separated (at specified R_s) over the "path length" of a chromatogram (or elution volume range V_{max} - V_{min}) in the separation system

***Taken from J. Calvin Giddings, Unified Separation Science, pg. 105 and references cited therein**

Peak Capacity (Z_p) - Isocratic Elution*

$$Z_p = \frac{\sqrt{N}}{6R_s} ln\left(1 + k'_{last}\right) + 1$$

Z_p : peak capacity (for uniform distribution in chromatographic space)
k'_{last} : retention factor of the last peak
R_s : required resolution (base line separation: $R_s \rightarrow 1.5$)

LC column, N = 25,000, k' = 20, Z_p = calculate

***Taken from J. Calvin Giddings, Unified Separation Science, pg. 106 and J.C. Giddings, Anal. Chem. 1967, 39, 1027–1028**

Peak Capacity (Z_p) – Gradient Elution

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Assuming linear solvent strength gradient*

$$Z_p = 1 + \frac{t_g}{1/n \Sigma_1^n w} = 1 + \frac{t_g}{w_{av}}$$

t_g = gradient time

w_{av} = average peak width

***U.D. Neue, Theory of Peak Capacity in Gradient Elution, J. Chrom. A, 1079, 153, 2005 and references cited therein.**

Peak Capacity (Z_p) in 1D HPLC

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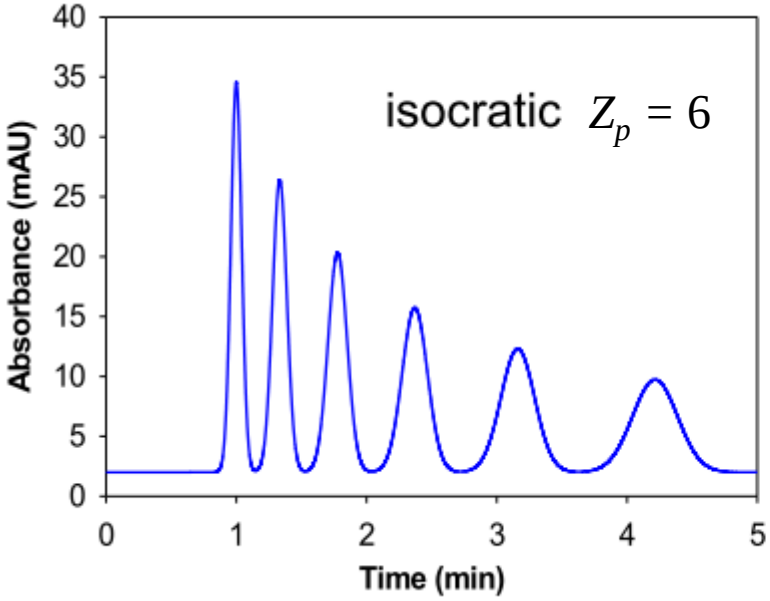
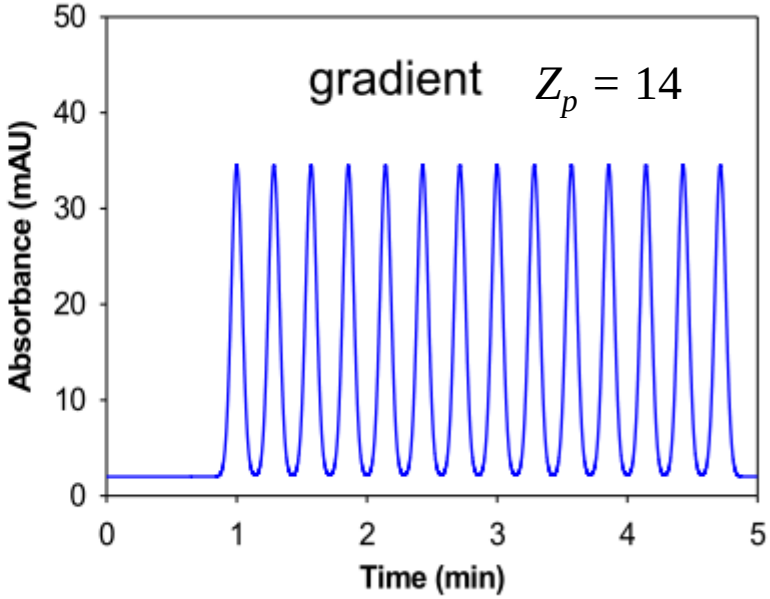
# of peaks separated with equal resolution



Assuming linear solvent strength gradient*



$$Z_p = \frac{\sqrt{N}}{6R_s} ln\left(1 + k_{last}\right) + 1$$

$$Z_p = 1 + \frac{t_g}{w_{av}}$$

Z_p : peak capacity
k'_{last} : retention factor of the last peak
R_s : required resolution (base line separation: $R_s \rightarrow 1.5$)

LC column, Z_p = 50, k = 10, N_{req} = calculate

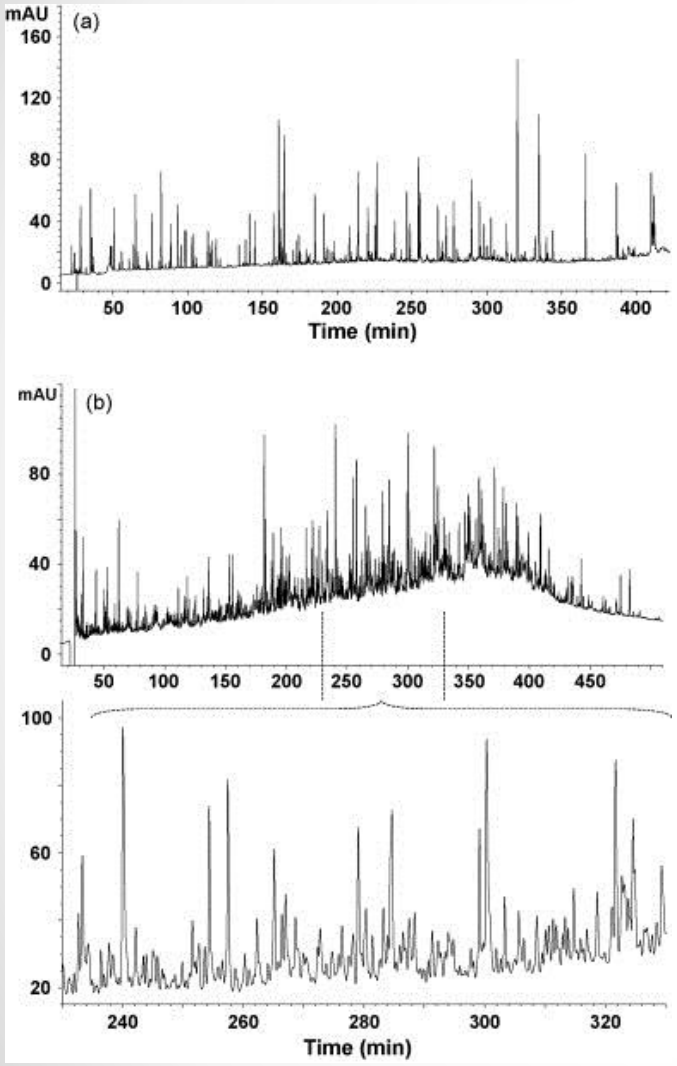
Constraints in Obtaining High Peak Capacity in 1D HPLC*

- Solutes do not elute in an equidistant way
- Congestion in the chromatographic space is leading to peak overlap/co-elution which becomes particularly relevant when there is a high number of solutes in the sample
- Peaks are not all the same size (height) thus the resolution criterion R_s = 1 may not show two maxima which only works for equal size peaks

***G. Guiochon, Journal of Chromatography A, 1126 (2006) 6–49**

Peak Capacity (Z_p) in 1D HPLC

Practical Example



BSA (a) and a depleted human serum tryptic digest (b) on 8 250× 2.1 mm ID × 5 µm Zorbax SB300-C18 columns.

Temperature 60 °C.

Mobile phase A 2% ACN, 0.1% TFA and mobile phase B 70% ACN, 0.1% TFA. Gradient slope 0.135% B/min, flow rate 200 µL/min.

Detection wavl. 214 nm

P. Sandra, G. Vanhoenacker, J. Sep. Sci., 30 (2007), p. 241

Constraints in Obtaining High Peak Capacity

- Solutes do not elute in an equidistant way
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Giddings et al. have assumed that the occurrence of a peak in the chromatographic space (from t_0 till t_{last}) is a randomly appearing discrete event (Poisson distribution). Depending on the width of each peak (which determines the peak capacity) and the number of solutes n_c in the sample, the number of solutes ***s*** that elute as a single band is given by:

$$s = n_c \exp\left(\frac{-2n_c}{Z_p}\right)$$

In the example before and n=10

*** J. C. Giddings, Unified Separation Science, pg. 131 and references cited therein**
J. C. Giddings & J. Davis, Anal. Chem., 55, 418 (1983); 57, 2168 (1985)

Peak Capacity (Z_p) in 1D HPLC

Davis–Giddings Statistical Model of Peak Overlap

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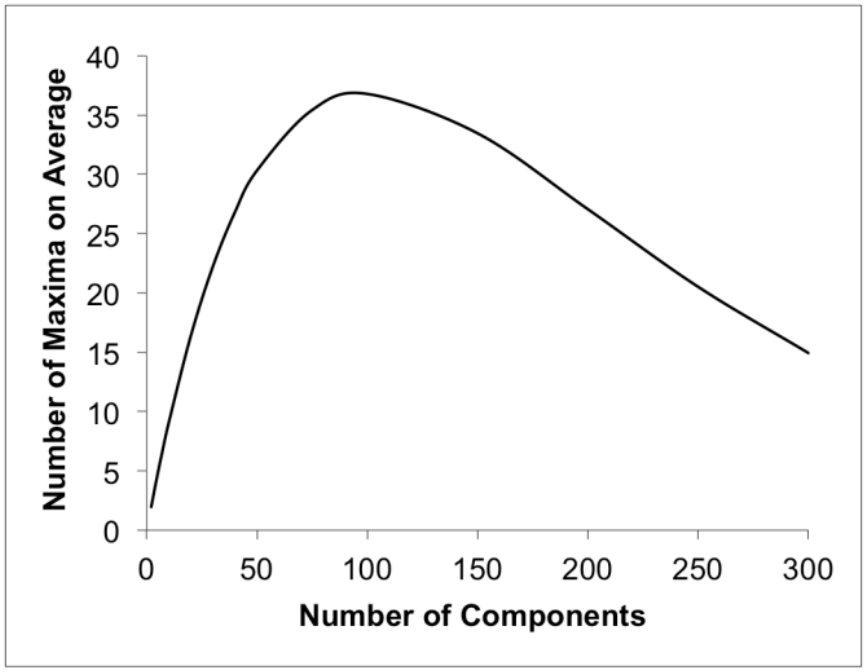
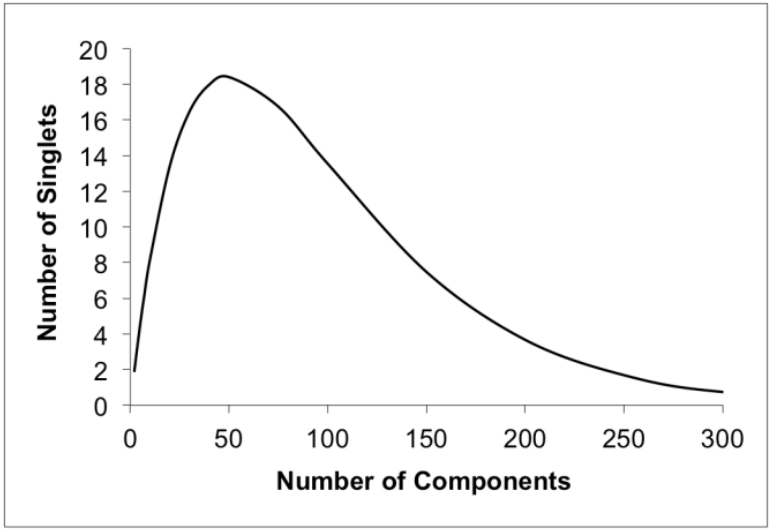
# of observed peaks (Z_p =100)



# of pure single peaks (Z_p =100)



$p_{max} = 0.37\ Z_p$

$s_{max} = 0.18\ Z_p$

Calculate

J. M. Davis and J. C. Glddings, Anal. Chem. 1983, 55, 418-424

Why do Multidimensional HPLC?

- 1D HPLC does not give enough resolution to deal with complex samples (n_c >> 50)
- Sample fingerprinting, classification, identification of contaminants, source of origin determination detection
- Essential for “non-targeted” analysis (e.g. life science research)
- Targeted analysis to isolate solutes from a complex matrix

Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept

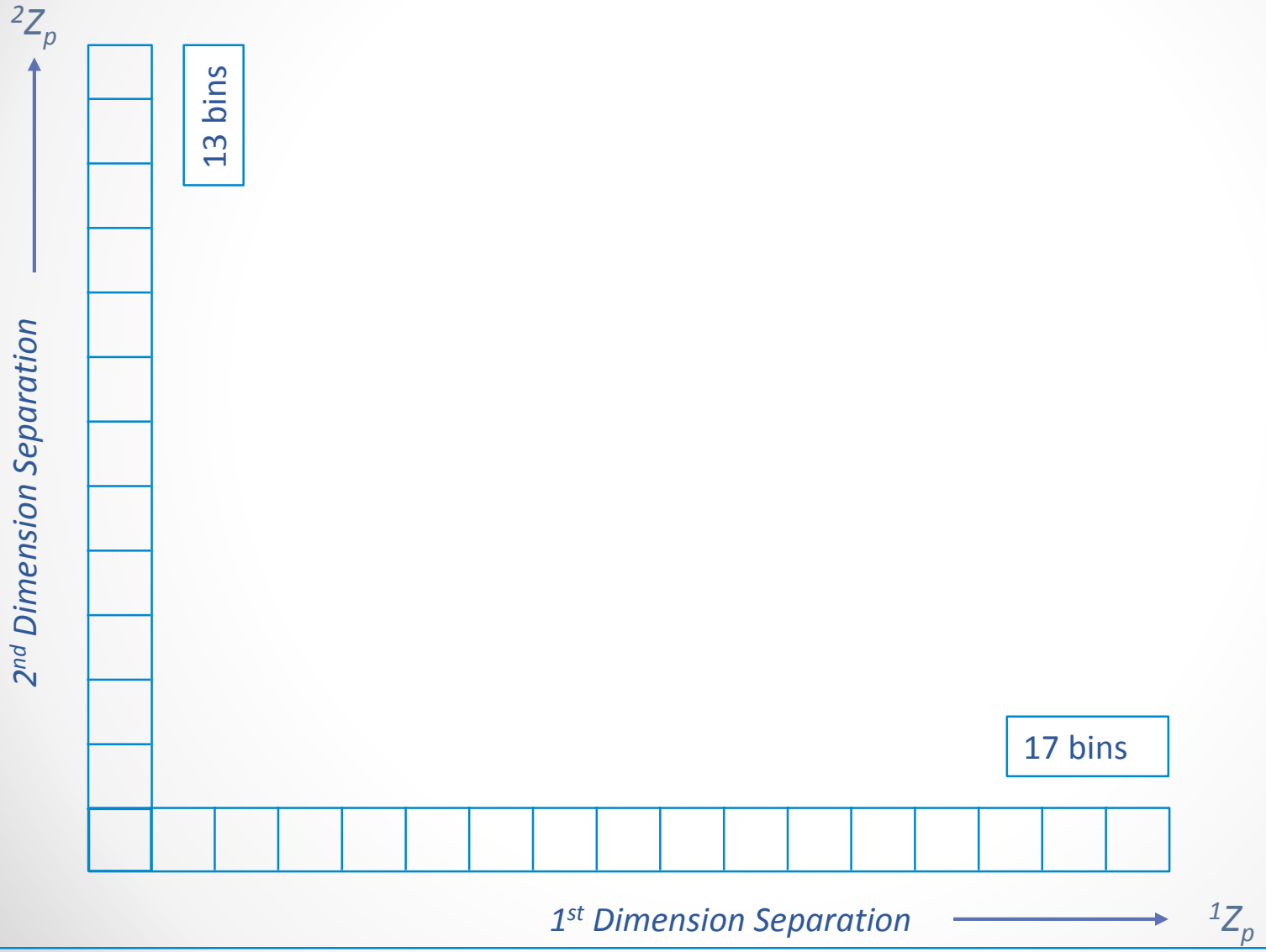
Microscale Separations and Bioanalysis

1st Dimension Separation →

Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept

Microscale Separations and Bioanalysis



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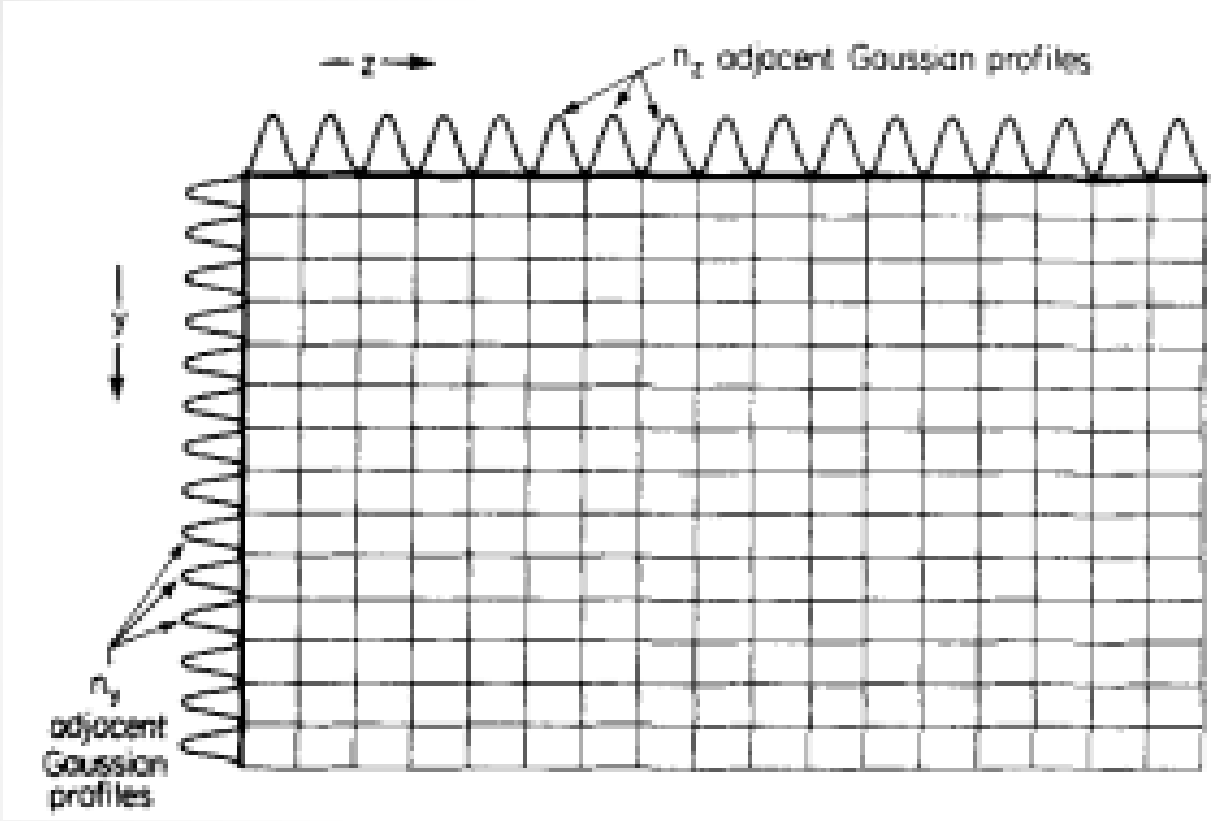
Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept

Microscale Separations and Bioanalysis



Theoretically:

$$^{2D}Z_p = {}^1Z_p \; x \; {}^2Z_p$$

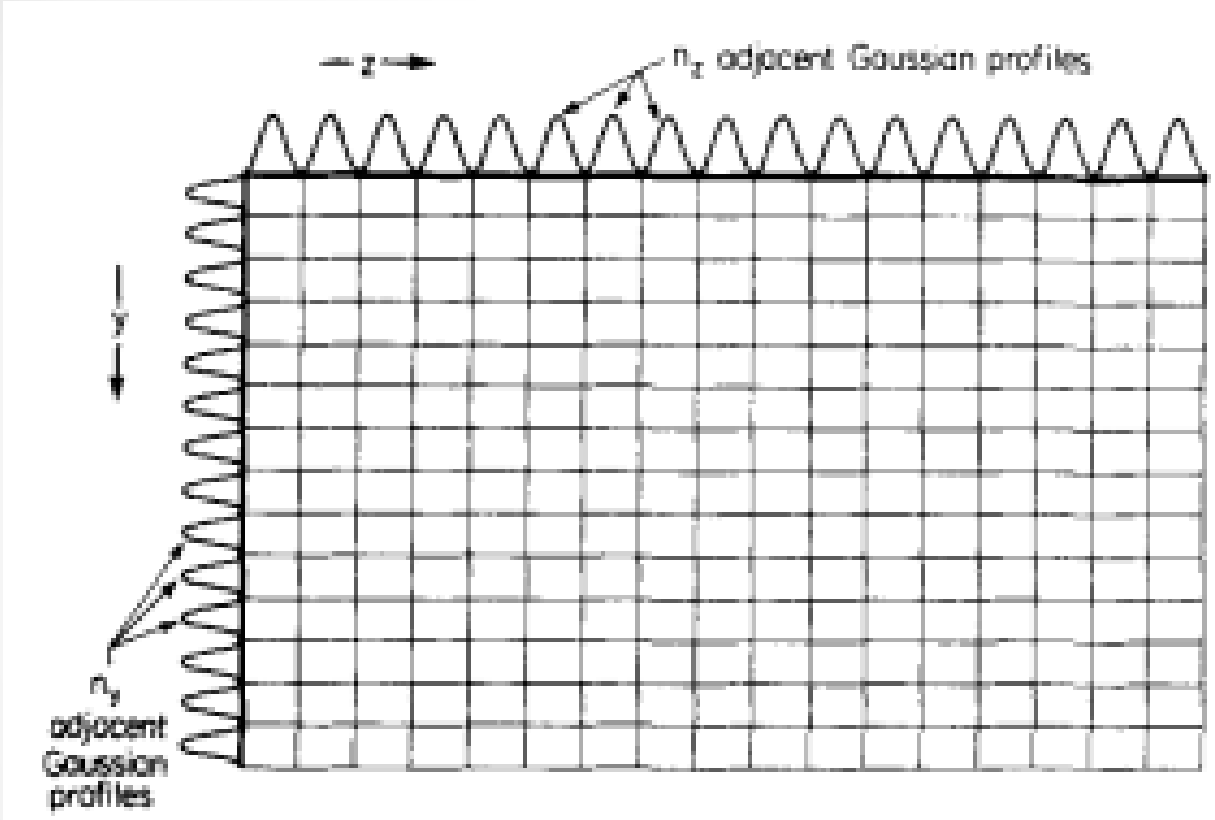
The Giddings "Product Rule"

Giddings, J. C. J. High Resolut. Chromatogr. 1987, 10, 319-323

Peak Capacity in Comprehensive 2DLC

The geometric orthogonality concept

Microscale Separations and Bioanalysis



In this case $^{2D}Z_p$= 17x14 = 238!
For 1D separation, N_{req} = calculate

Theoretically:

$$^{2D}Z_p = {}^1Z_p \; x \; {}^2Z_p$$

The Giddings "Product Rule"

Giddings, J. C. J. High Resolut. Chromatogr. 1987, 10, 319-323

Peak Capacity in Comprehensive 2DLC

Giddings Criteria for the Product Rule

- **ORTHOGONALITY:**
  "The components of a mixture are subjected to two or more separation steps in which their displacements depend on different factors."
  - The retention of the sample solutes must be controlled by two (or more) different physical-chemical properties and the two separation systems must separate the species by different mechanisms. The retention of the component is describe by two or more retention times (by 2 dimensions)
- **SAMPLING:**
  "When two components are substantially separated in the first step, they remain separated until the completion of the second separation step."
  - Width of the sample (in time or volume units) from first separation
  - Once the solutes are separated there must be no remixing (peak broadening) induced by doing the second separation

J.C. Giddings in "Multidimensional Separations", H. J. Cortes (ed.), vol. 50 in Chromatographic Science Series, Marcel Dekker, 1990.

Requirements to Achieve Theoretical Peak Capacity in Comprehensive 2DLC

1. **ORTHOGONALITY** of separation mechanisms – This is a requirement imposed mostly on the **stationary phase** chemistry.
2. Peaks must cover **ENTIRE** separations "space".
3. Separation gained in one dimension cannot be diminished by separation in the other dimension. Must sample **FAST!**

$$ {}^{2D}Z_p = {}^{1}Z_p \times {}^{2}Z_p \times \frac{1}{\langle \beta \rangle} \times f_{coverage} $$

Under Sampling Correction

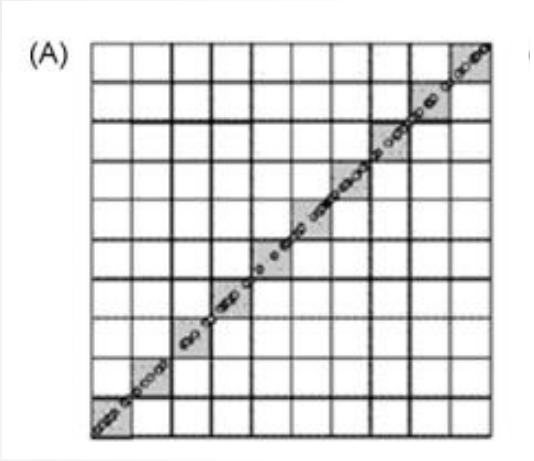
Orthogonality

What is the most important factor?
How can we improve it?

Davis, J. M.; Stoll, D., R.; Carr, P. W. *Anal. Chem.* 2008, 80(2), 461-473
Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990

Orthogonality in 2D LC

Orthogonality in Comprehensive 2DLC



(A) Non-orthogonal system, ^{1}D column is identical with ^{2}D column. Area coverage represents 10% orthogonality.

M. Gilar et al. Anal. Chem., 77, 6426 (2005)

Separation Space Utilization by Orthogonal and Correlated Mechanisms

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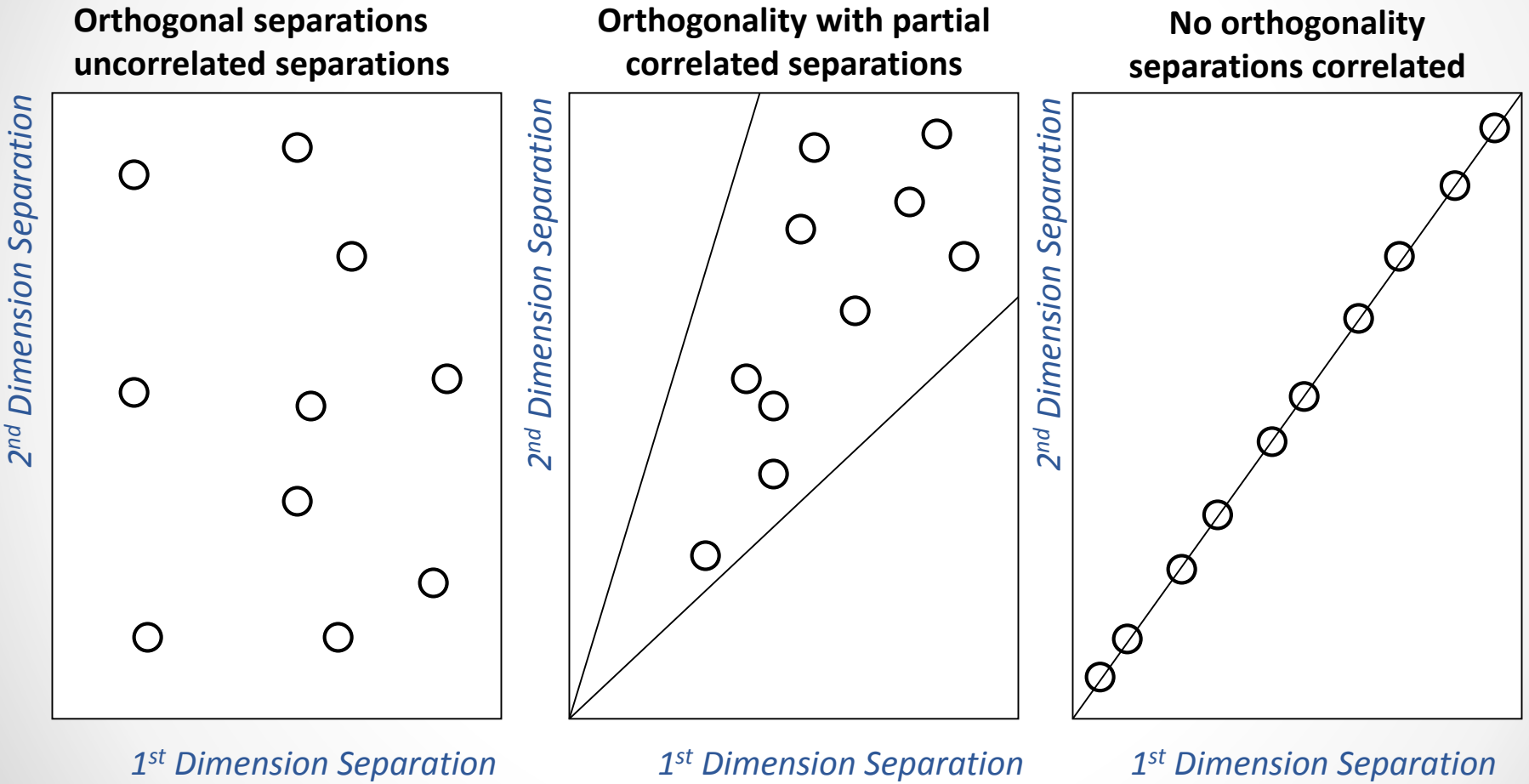


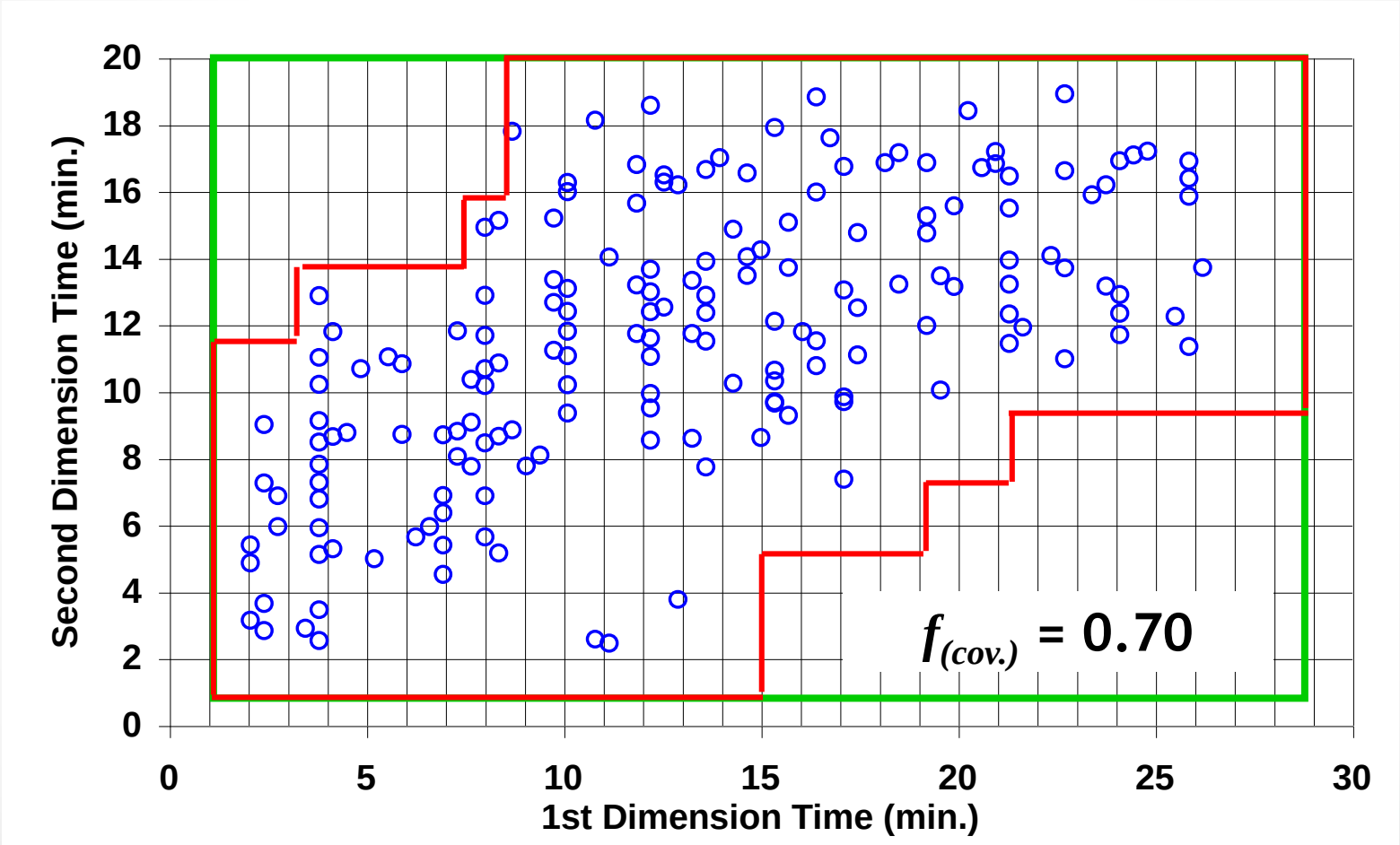
Typical

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Fractional Utilization of 2D Space

(Stoll modified Gilar method)

Microscale Separations and Bioanalysis



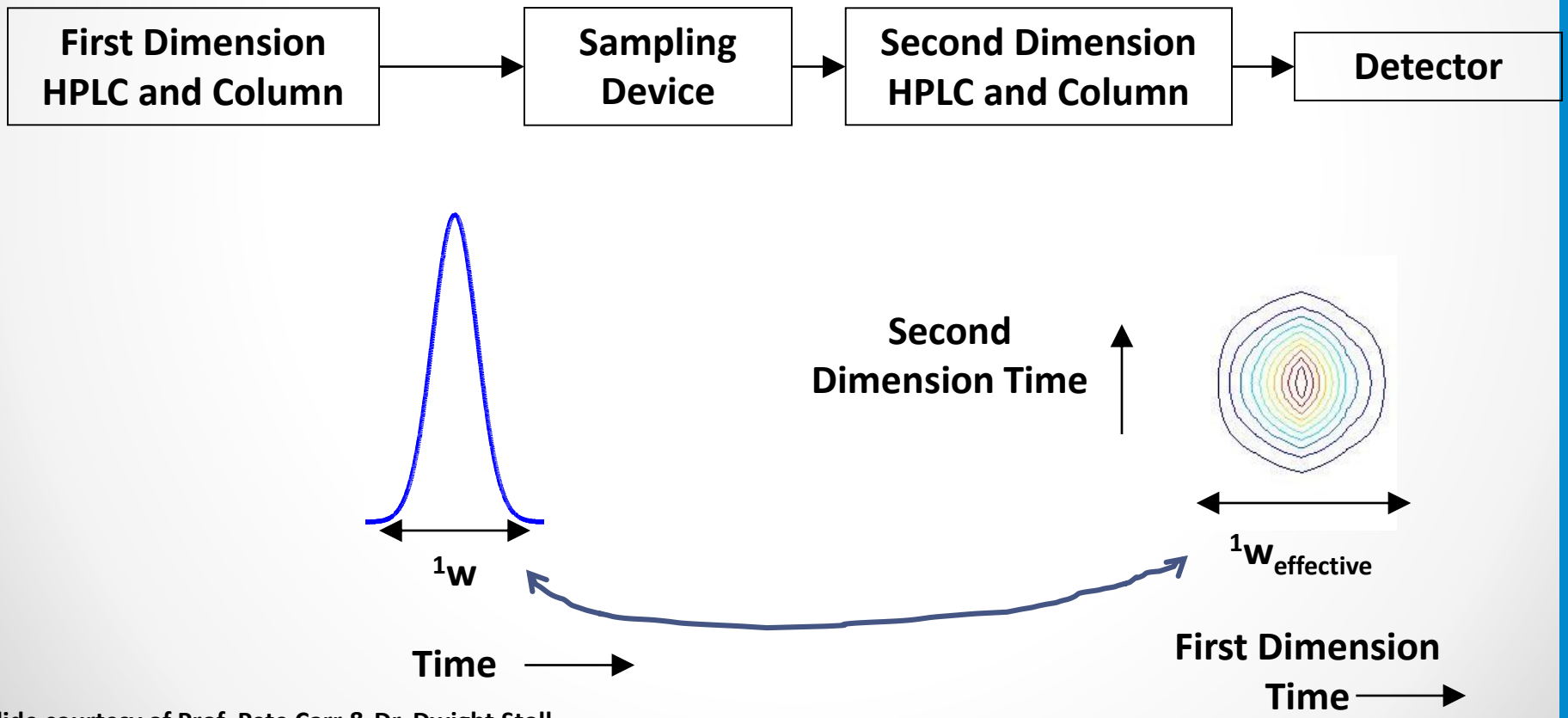
Stoll, D.R., et al. *Anal. Chem*. 2008, 80, 268-278
Gilar, M. et al. *Anal. Chem.* 2005, *77*, 6426-6434.

The Sampling Problem in 2D LC

Giddings's Second Criterion

Microscale Separations and Bioanalysis

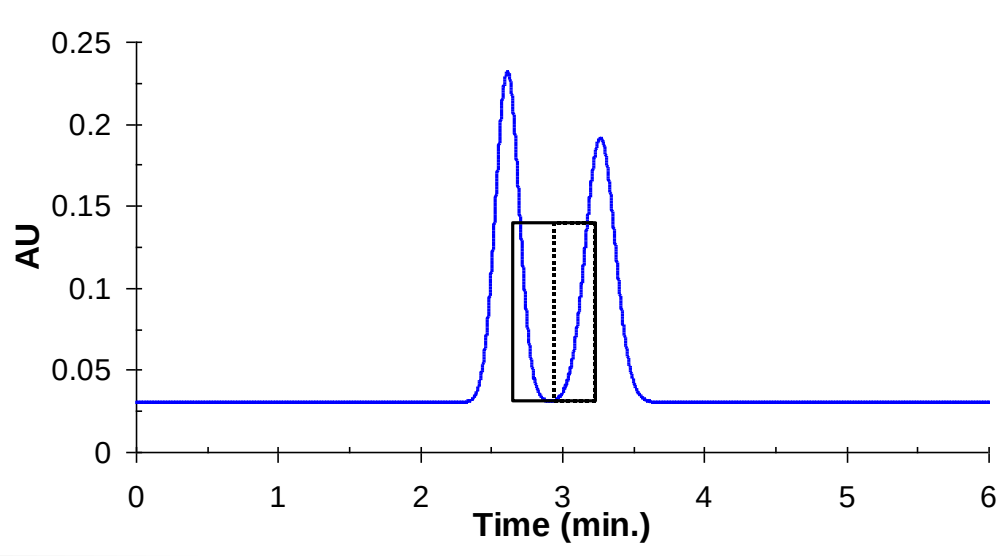
The width of a peak observed in a 2D chromatogram in the direction of the first dimension axis after sampling is effectively broader than the width of the peak that elutes from the first dimension column before sampling.



Slide courtesy of Prof. Pete Carr & Dr. Dwight Stoll

The Undersampling Problem*

The Murphy-Schure-Foley Criterion**



Clearly if we take a sample as indicated and inject it into a second dimension we will partially “undo “ the separation already accomplished in the first dimension.

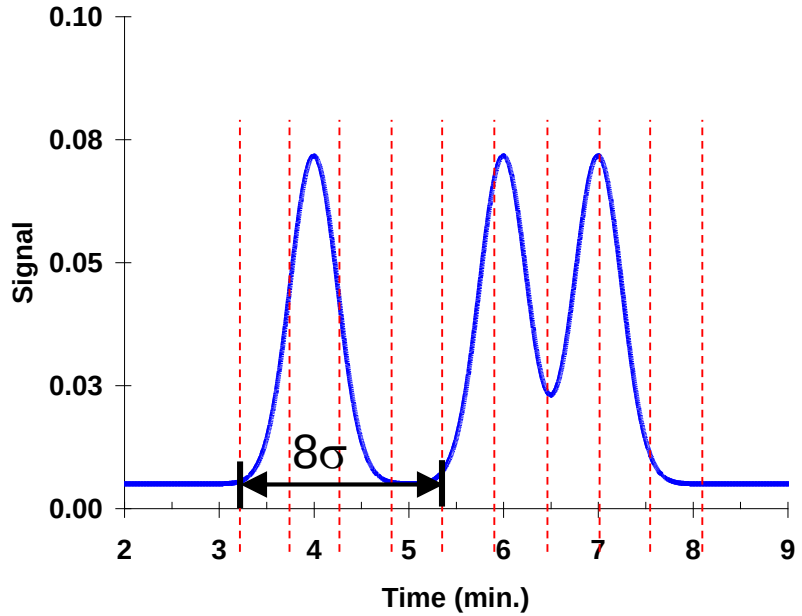
According to M-S-F** **one needs to take at least 4 samples** across the 8σ base width of each first dimension peak **to minimize the effect of undersampling**.

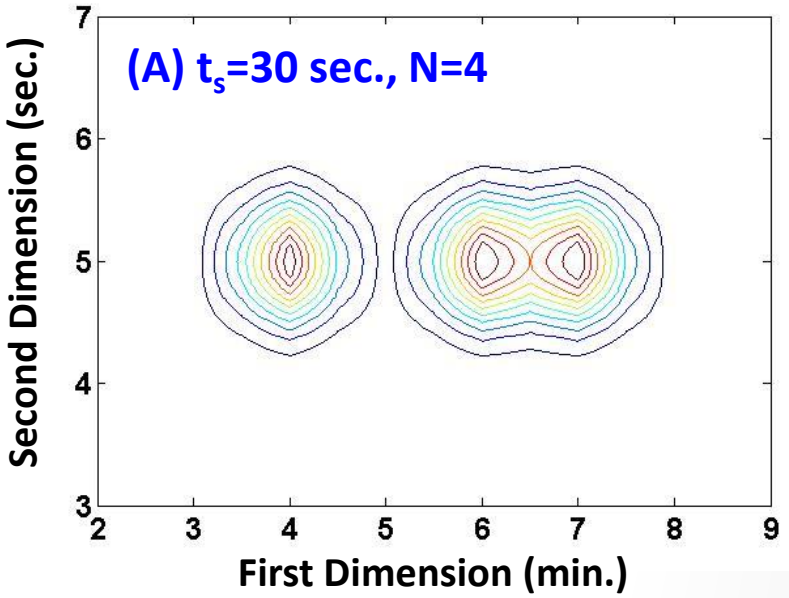
***Slide courtesy of Prof. Pete Carr & Dr. Dwight Stoll**

****Murphy, R. E.; M. R. Schure; J. P. Foley Anal. Chem., 1998; Vol. 70, pp 1585**

The Undersampling Problem

The Murphy-Schure-Foley Criterion





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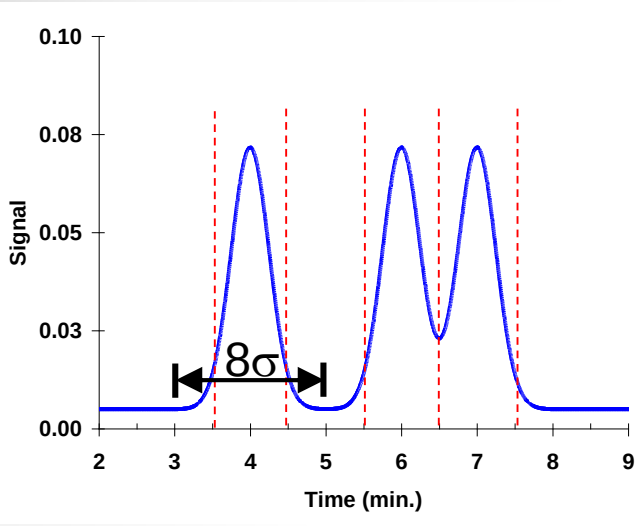
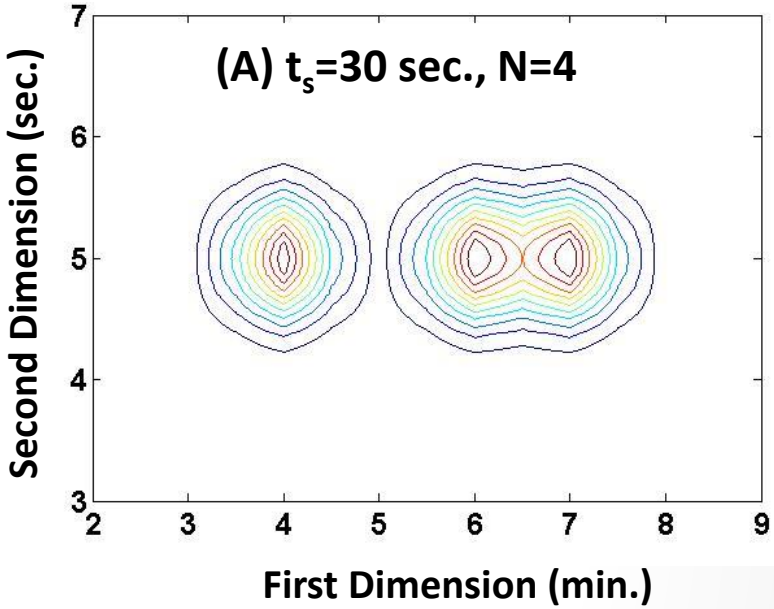
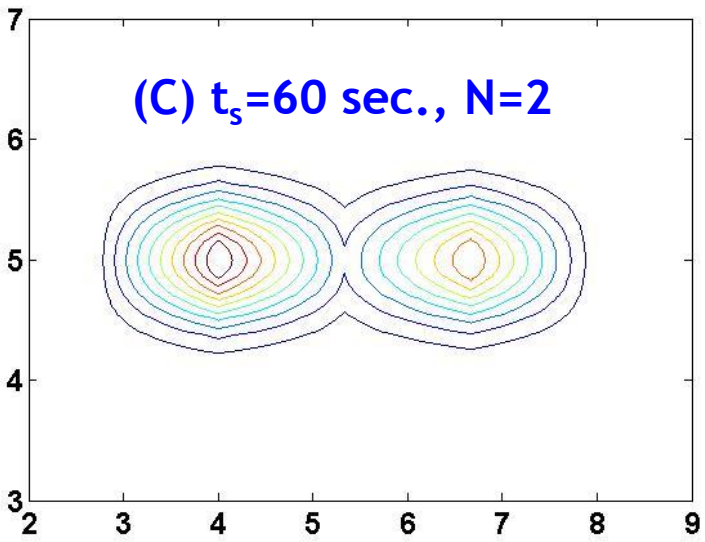
The Undersampling Problem

The Murphy-Schure-Foley Criterion

Microscale Separations and Bioanalysis







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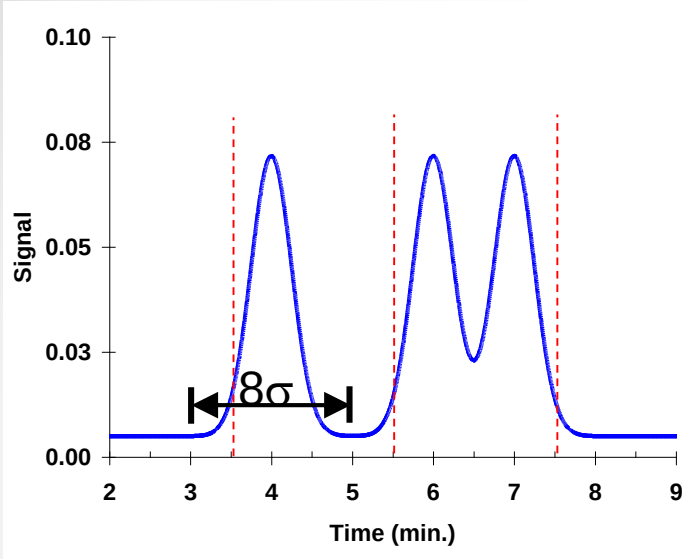
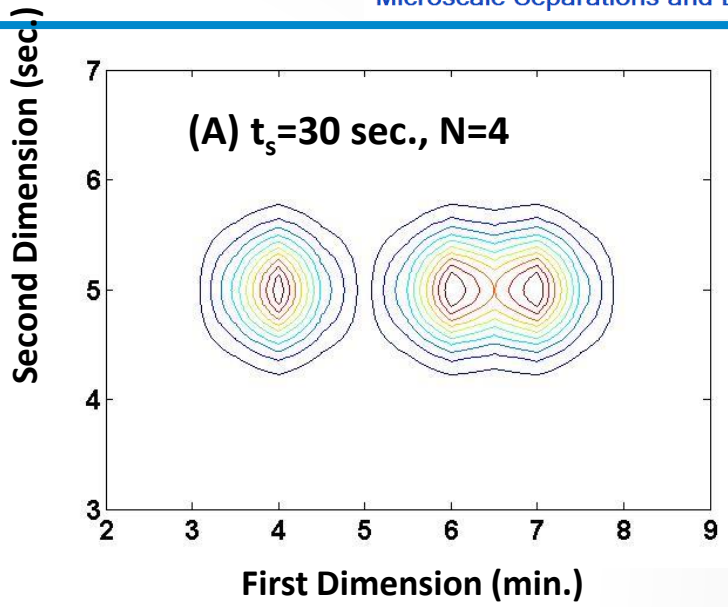
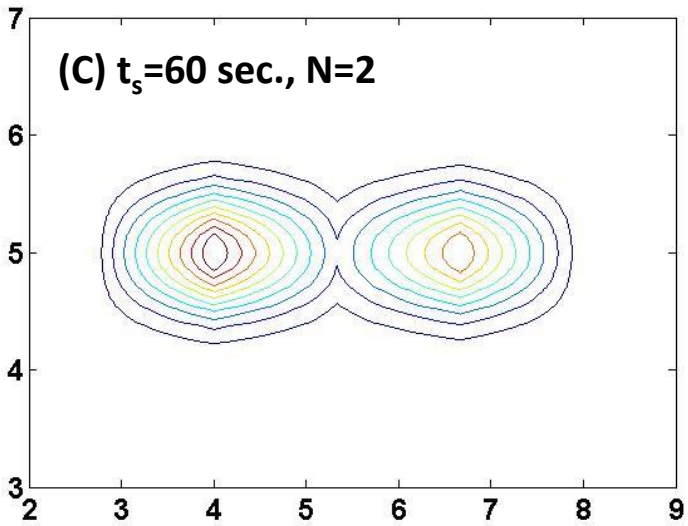
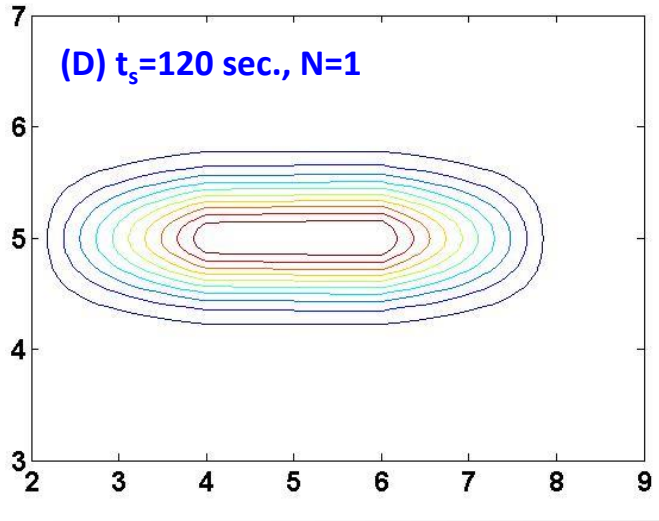
The Undersampling Problem

The Murphy-Schure-Foley Criterion







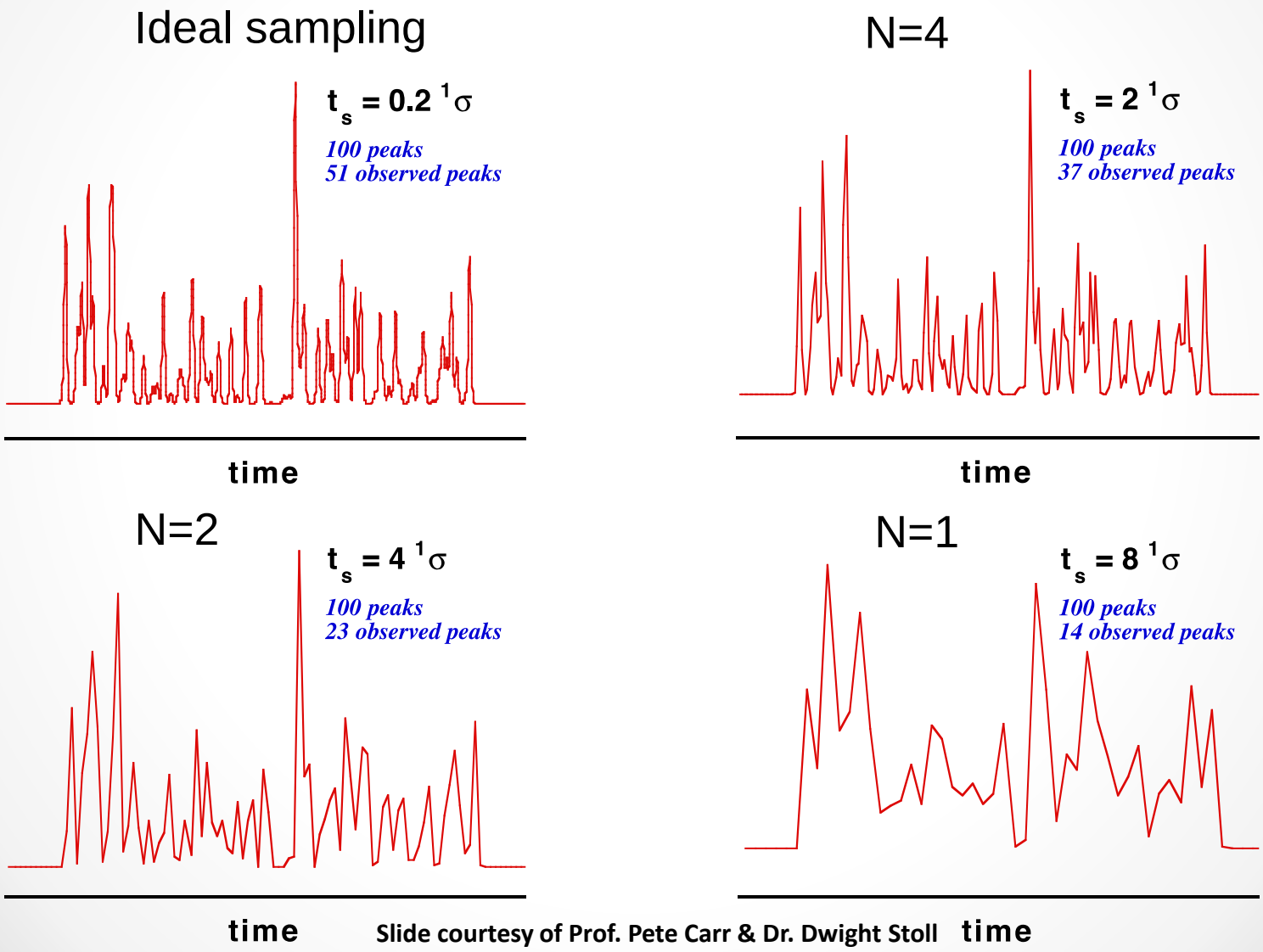


Slide courtesy of Prof. Pete Carr & Dr. Dwight Stoll

The Undersampling Problem

Alternative View of Undersampling the First Dimension

Microscale Separations and Bioanalysis



Slide courtesy of Prof. Pete Carr & Dr. Dwight Stoll

Peak Capacity in Comprehensive 2DLC

"Effective" Peak Capacity

Microscale Separations and Bioanalysis

$$^{2D}Z_p = {}^{1}Z_p \times {}^{2}Z_p \times \frac{1}{\langle\beta\rangle} \times f_{coverage}$$

$$<\beta>= \sqrt{\left(1+0.214.\left(\frac{t_s}{<{}^{1}\sigma>}\right)^2\right)}$$

Under Sampling Correction

Orthogonality

t_s sampling time, $^{1}\sigma$ average width 1st dimension peaks

1. <β> average correction for under sampling*
2. $f_{coverage}$ corrects for incomplete use of the separation space.

What is the most important factor?
How can we improve it?

***D.R. Stoll et al., Anal. Chem. 2008, 80, 268-278; Davis, J. M. Stoll, D., R. Carr, P. W. *Anal. Chem.* 2008, 80(2), 461-473; Giddings, J. C. *Multidimensional Chromatography: Techniques and Applications*; Marcel Dekker: New York, 1990**
Slide courtesy of Prof. P. Carr & Dr. D.R.Stoll

Peak Capacity in Comprehensive 2DLC

Implications of <β>

Microscale Separations and Bioanalysis

ROZING.COM Consulting

- We want to make the sampling time short.
- In LC x LC ${}^1t_{sample} = {}^2t_{cycle}$
- Prefer ${}^1t_{sample} < {}^2t_{cycle}$ (under fill the sample loop!)
- ${}^2t_{cycle} = {}^2t_{gradient} + {}^2t_{re\text{-}equilibration}$
- Don't make ${}^1t_{sample}$ too short since 2D separation peak capacity decreases if ${}^2t_{gradient}$ decreases
- ➔Fast, very high efficiency separation in the 2nd dimension

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Requirements to the 1st Dimension Separation

Dimensions, Isocratic or Gradient Elution

1st Dimension Separation

Requirements

Microscale Separations and Bioanalysis

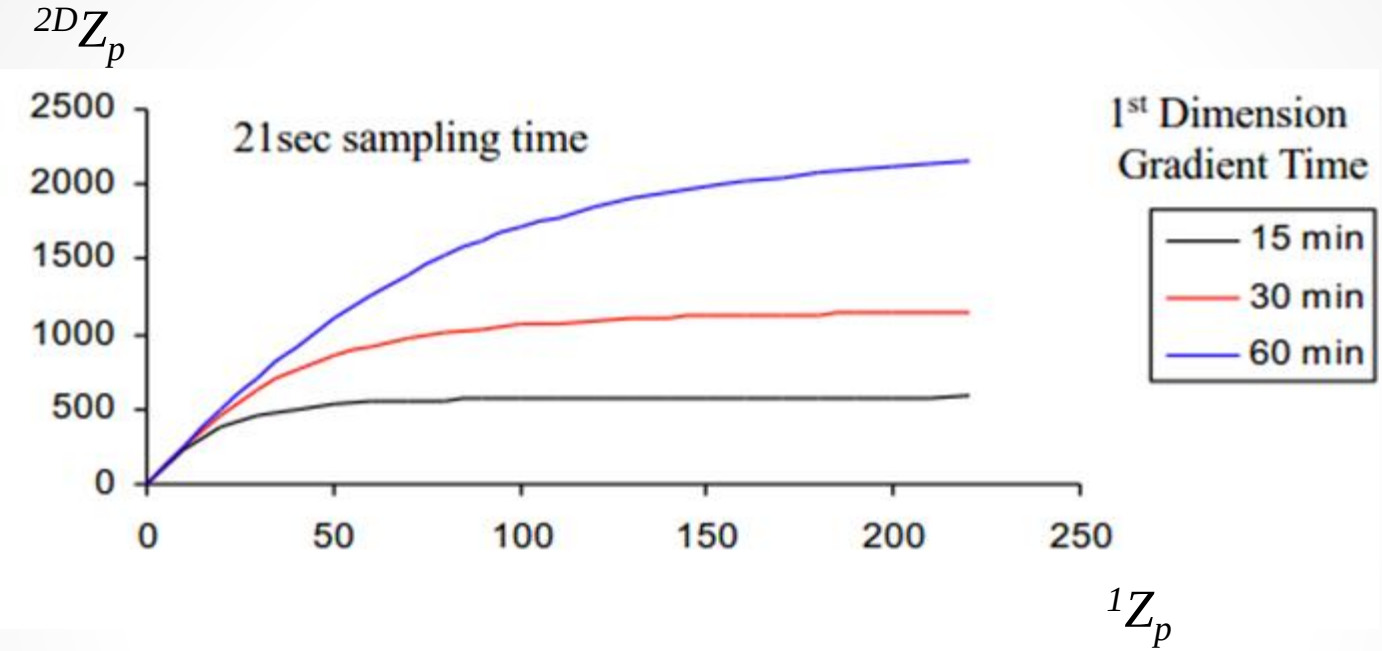
- Narrow and long columns are preferred
- Use low flow rate where possible
  - 1D Flow Rate = 200 µL/min, Sampling Time = 20 s
    ➔ Volume Injected to 2D Column = 67 µL
- Use stationary phase that can tolerate extreme conditions (e.g. low or high pH)
- Isocratic separation or use a slow gradient separation
  - Peak width in isocratic separation is not constant!! may lead to under sampling early and over sampling late in the chromatogram

Slide courtesy of Prof. P. Carr & Dr. D. Stoll

Peak Capacity in Comprehensive 2DLC

Influence of 1st dimension gradient steepness

Microscale Separations and Bioanalysis



L.W. Potts, D.R. Stoll, X. Li, P.W. Carr J. Chrom. A (2010), 1217, 5700-5709

Requirements to the 2nd Dimension Separation

Requirements to the 2nd Dimension Separation

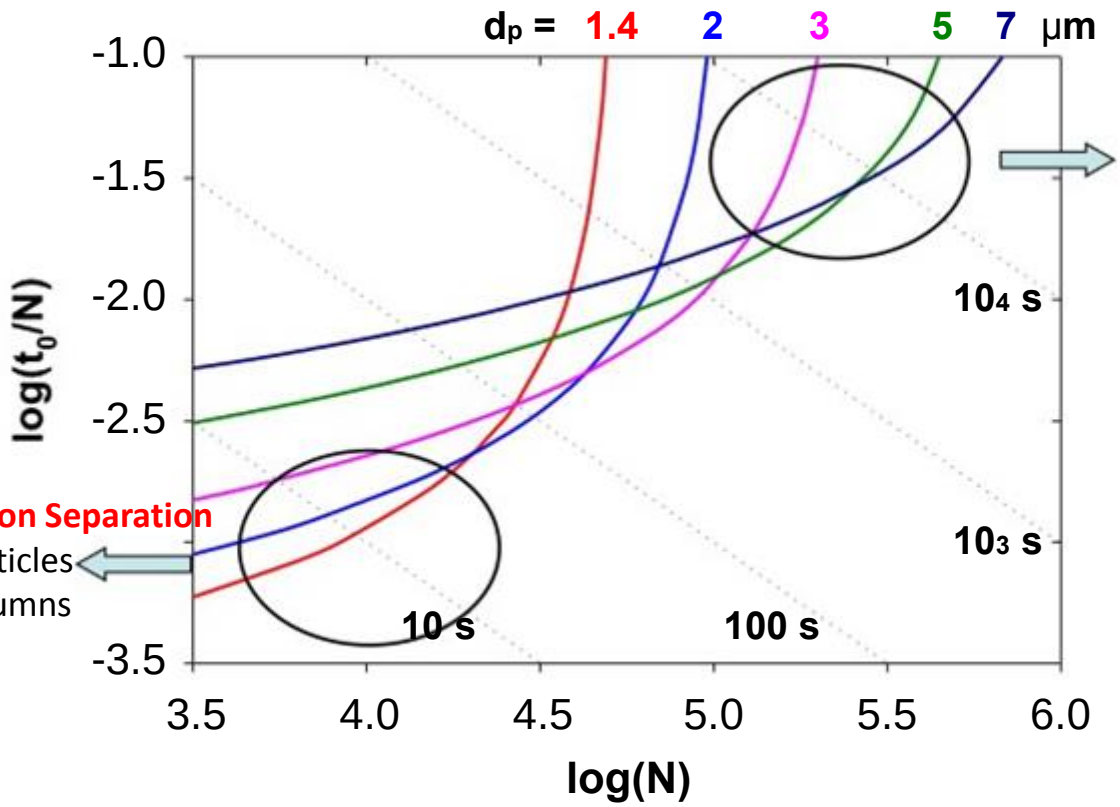
Microscale Separations and Bioanalysis

- Must be very high speed separation(UHPLC!!)
- Isocratic or gradient separation
- 2nd dimension stationary phase should provide analyte focusing
- Stable column with minimal retention time drift and have excellent longevity (e.g. at low pH, high pressure or temperature)

2nd Dimension Separation

Influence of Particle Size – Poppe Plot for Isocratic Separation

Microscale Separations and Bioanalysis



First Dimension Separation

- Larger particles
- Long columns
- Long run time

2nd Dimension Separation

- Small particles
- Short columns

ΔP_{max} = 400 bar

Knox equation

A = 1.0, B = 1.5, C = 0.05

$D_m = 1 \times 10^{-5}$ cm²/s

$\eta = 0.001$ *Pa/s*

- Use short columns with very small particles for ultra fast separations
- Work at ultra high pressure

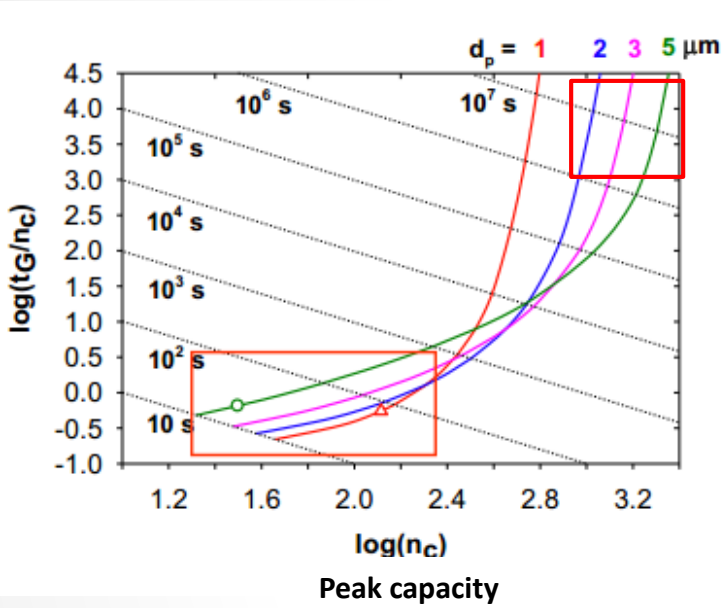
Slide courtesy of Prof. Pete. Carr & Dr. Dwight Stoll

2nd Dimension Separation

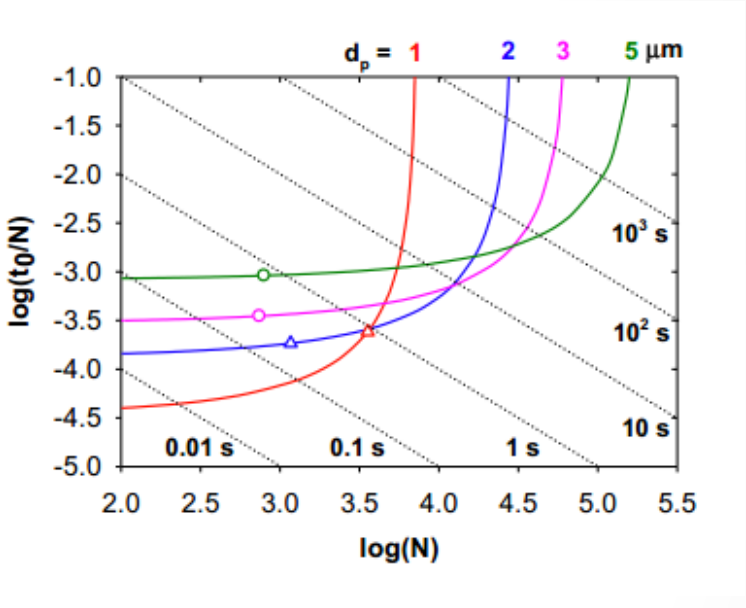
Influence of Particle Size – Poppe Plot for Gradient Separation

Microscale Separations and Bioanalysis

Gradient Poppe Plot for 11 Peptides



Isocratic Poppe Plot for Alkylphenone



ΔP_{max} = 400 bar D_m = 1 ×10-5 cm²/s, η = 0.69 *cP*

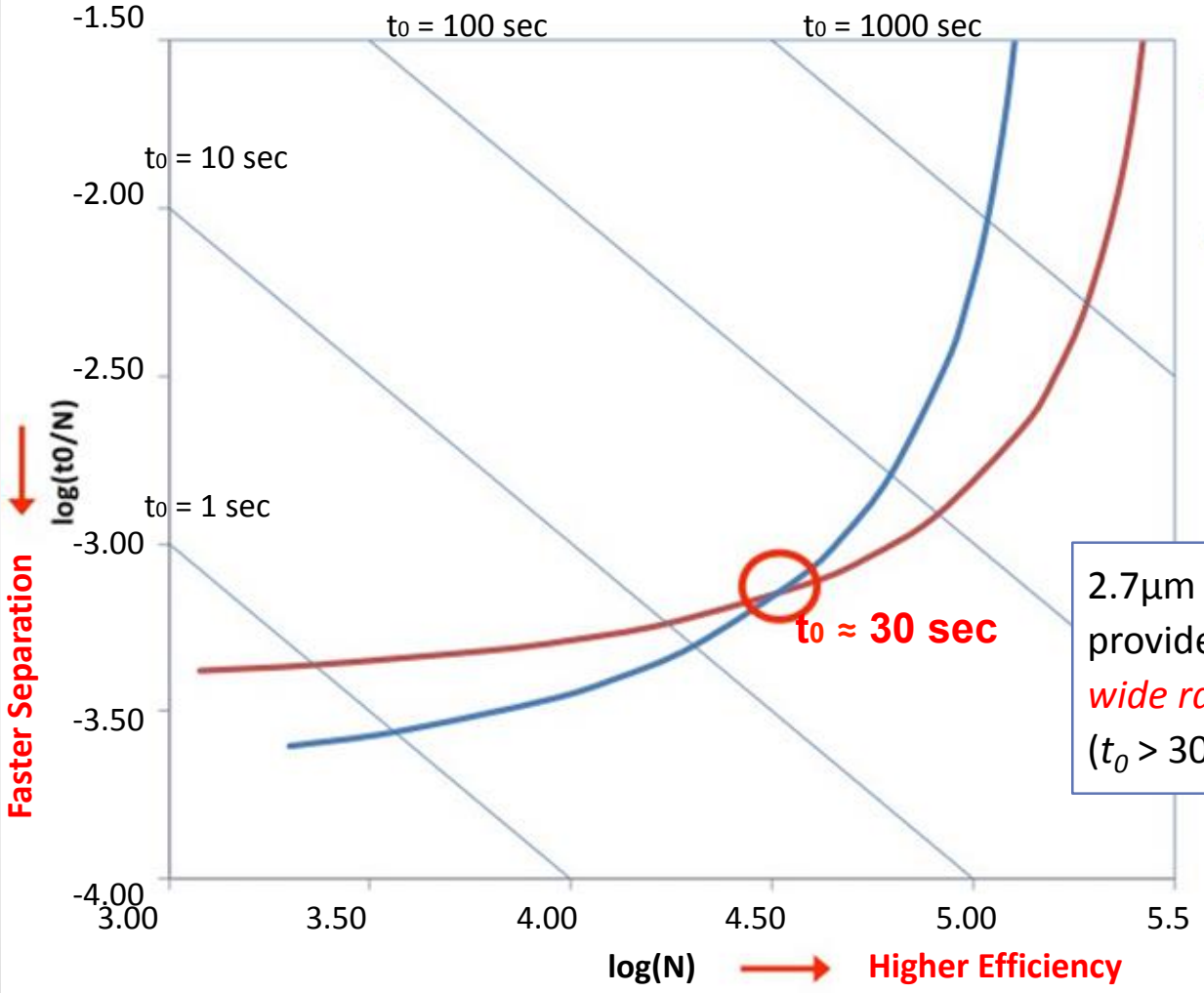
Isocratic and gradient Poppe plots lead to qualitatively the same conclusions on effect of particle size on peak capacity

Wang, X.; Stoll, D. R.; Carr, P. W.; Schoenmakers, P. J. J. Chromatogr., A 2006, 1125, 177

2nd Dimension Separation

Sub-2-µm Totally Porous vs Superficially Porous Particles

Microscale Separations and Bioanalysis

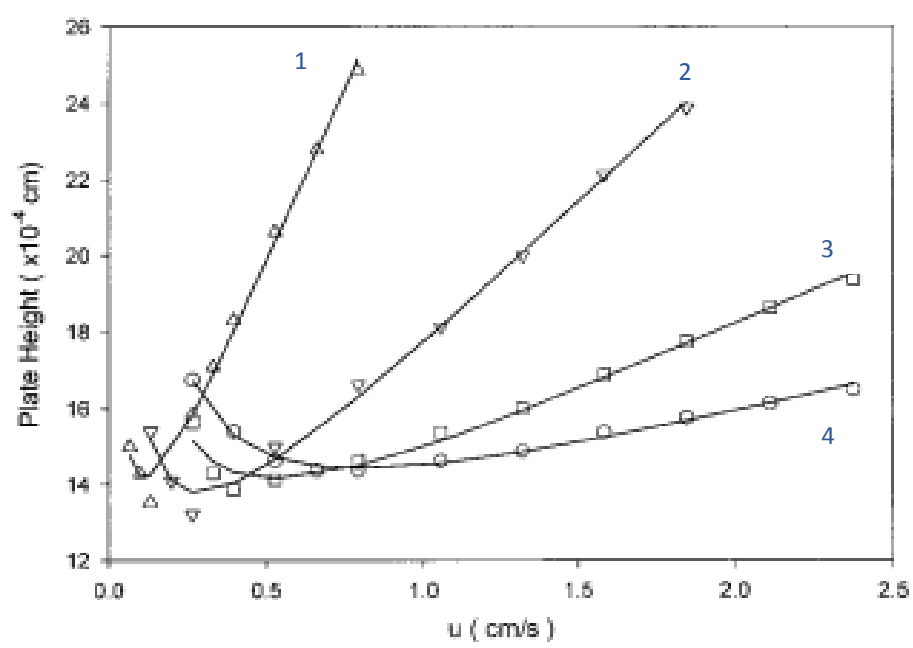


2.7µm superficially porous particles provide best separation in *a very wide range of analysis time* (t_0 > 30 sec, or t_R > 3 min)

Wang et al J. Chromatogr. A 2012, 1228, 72-88

2nd Dimension Separation

Effect of Temperature*



High temperature for the 2nd dimension separation

- $u_{0,opt}$ increases
- C-term flattens

Plate height vs linear velocity at various temperatures for well-retained solutes;
1, 25 °C (decanophenone, k 12.2)
2, 80 °C (dodecanophenone, k 7.39)
3, 120 °C (tetradecanophenone, k 12.3)
4, 150 °C (tetradecanophenone, k') 7.00).

***P. Carr et al., Anal. Chem. 2000, 72, 1253-1262**

End of Part 1